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CELL WALL LIPOPOLYSACCHARIDES OF 'YERSINIA PESTIS': CHEMICAL AN--ETC(U)
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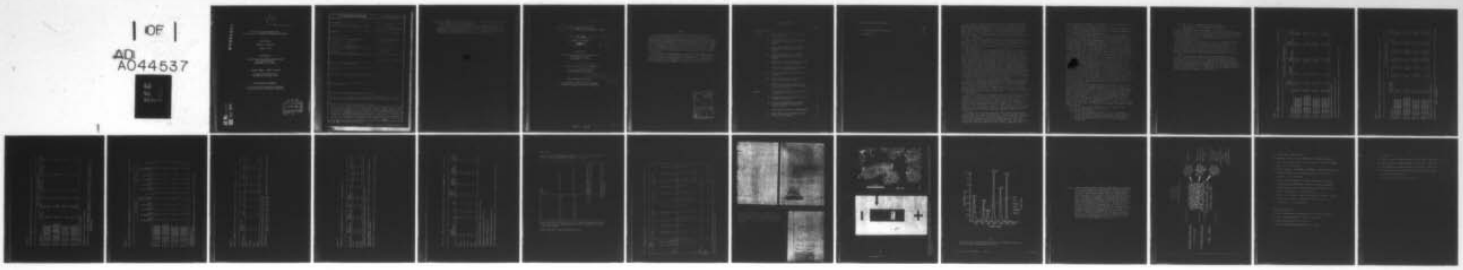

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Cell Wall Lipopolysaccharides of *Yersinia pestis*: Chemical and Serological Studies

Final Report

Thomas G. Tornabene

January, 1977

Supported by:

United States Army Medical Research and
Development Command
Washington, D.C. 20314

Contract Number: DAMD17-74-C4040

Colorado State University
Fort Collins, Colorado 80523

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Cell Wall Lipopolysaccharides of *Yersinia pestis*: Chemical and Serological Studies		5. TYPE OF REPORT & PERIOD COVERED Final Report 12/1/73 to 12/31/76 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Thomas G. Tornabene		8. CONTRACT OR GRANT NUMBER(s) DAMD17-74-C4040
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Microbiology Colorado State University Fort Collins, Colorado 80523		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102B 3A161102B71Q.00.076
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command Washington, D.C. 20314		12. REPORT DATE January, 1977 13. NUMBER OF PAGES 26
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

The findings in this report are approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Yersinia pestis, *Pseudomonas maltophilia*, Lipopolysaccharides, Acidic polysaccharides, Lipids, proteins, glycoproteins, capsule, cell wall, virulence

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The research program characterized the macromolecular structures comprising the envelope of *Yersinia pestis*, the causative agent of bubonic plague. The unit protein structures of the macrocapsule, acidic polysaccharide of microcapsule, and lipopolysaccharide of cell wall were elucidated and formulated into a model postulating the structural relationship of each entity. Studies on biochemical events and cellular factors were conducted and associated with the expression of virulence. A synergistic event between *Pseudomonas maltophilia* →

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20. or its cellular exogenates and *Yersinia pestis* greatly enhanced the expression of virulence of the plague bacilli.

The lipopolysaccharide study showed that the lipopolysaccharide contents of *Yersinia pestis* strains were chemically similar and cannot be employed for differentiating strains of plague bacilli. This program also established that serological typing of *Y. pestis* cells will not be accomplished by a simple exploration of somatic cellular entities.

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(6) Cell Wall Lipopolysaccharides of *Yersinia pestis*: Chemical and Serological Studies.

(9) Final Report. 1 Dec 73-31 Dec 76,

(10) Thomas G. Tornabene

(11) January, 1977

(12) 26p.

Supported by:

United States Army Medical Research and
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(16) 3A161102B71Q

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Abstract

The research program characterized the macromolecular structures comprising the envelope of *Yersinia pestis*, the causative agent of bubonic plague. The unit protein structures of the macrocapsule, acidic polysaccharide of microcapsule, and lipopolysaccharide of cell wall were elucidated and formulated into a model postulating the structural relationship of each entity. Studies on biochemical events and cellular factors were conducted and associated with the expression of virulence. A synergistic event between *Pseudomonas maltophilia* or its cellular exogenates and *Yersinia pestis* greatly enhanced the expression of virulence of the plague bacilli.

The lipopolysaccharide study showed that the lipopolysaccharide contents of *Yersinia pestis* strains were chemically similar and cannot be employed for differentiating strains of plague bacilli. This program also established that serological typing of *Y. pestis* cells will not be accomplished by a simple exploration of somatic cellular entities.

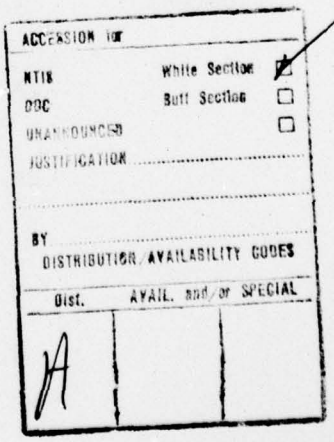


TABLE OF CONTENTS

Table of Contents (Continued)

The research program had the objectives of elucidating the detailed chemical composition and serological properties of proteins, polysaccharides and lipopolysaccharides of *Yersinia pestis*. These objectives have essentially been accomplished. A considerable quantity of fundamental data on cellular composition of plague bacillus is now realized. The ultimate goal of acquiring detailed data for the establishment of a system for chemo- or serological-typing of strains of plague bacilli has not been accomplished.

The studies now here summarized are those on somatic structures, namely macrocapsule, microcapsule, and outer cellular membrane, of five strains of *Y. pestis* and two strains of Pestoides. Attempts were made to present this summary program in a unified manner; even though it is somewhat naturally divided into specific studies dealing with respective cellular subfraction.

The composition of lipopolysaccharide (LPS) comprising the major portion of the outer cellular membrane received a great deal of attention. The chemical compositions are presented in Tables I-VI. The chemical entities comprising the LPS are essentially the same for all strains studied. The relative intensities of each entity are different; however, these same changes in relative intensities of a given composition from a single species were also observed when subjected to changes in age of cells, nature of cultivation medium, as well as to any physical chemical environmental changes. We have made extensive attempts at subfractionating LPS by column and thin-layer (Fig. 1) chromatography, sequential extractions, and so forth, to separate specific species of LPS, but to no avail. Chemical differences of each subfraction were observed only in the changes in relative intensities of the entities comprising the individual LPS subfractions. No specific serological differences could be detected between subfractionated, protein derivatized, cross-absorbed, and total LPS of the four *Y. pestis* strains studied.

There is no doubt that the LPS extracts consist of a variety of LPS species, but to a large part these exist only as degrees of aggregated forms and to a lesser degree those with varying ratios of hydrophobic ⟷ hydrophilic portions of LPS molecule. There are also definite indications for the existence of numerous additional oligosaccharides included within the cell wall structures but with compositions resembling fragments of the classical LPS molecule. No serological differences could be denoted.

During the course of our study with the LPS, we studied the detailed composition of the capsule surrounding and perhaps integrated with the cell wall. The macrocapsule is comprised of two identical proteins, one of which differs from the other by containing an oligogalactan. The physical and chemical properties of these proteins were described in detail and were subsequently published (J. Bacteriol. *117* (1974) 48). Two other chemical fractions, lipids and acidic polysaccharide, were also separated from the envelope of the cell. The lipids were fully characterized and described and also published (Biochim. Biophys. Acta. *306* (1973) 173).

A great deal of time has been spent on the acidic polysaccharide fraction (APS) and effort is now underway to obtain financial support to complete this work. The multispecies comprising the APS fraction contain specific components that will serologically cross-react with anti-F_1 and those that will cross-react with anti-LPS. The composition of the APS is hexoses, hexuronic acids, sulfates, phosphates, and a

small percentage of unknowns. The composition closely resembles specific heparin molecules and chrondoitin sulfates.

Through cultivation schemes and specific labelling procedures with ^{14}C, ^{32}P and ^{35}S atoms we have proven that *Y. pestis* cannot synthesize the APS in total and specifically incorporate and alter APS when they are available in the growth support medium.

In the course of the study we found that the APS was chemically similar to those that normally occur in all meat and agar preparations, respectively. However, we also found APS production by cells in synthetic media that originally contained no APS. In this synthetic media, through successive cultivations, we observed a contaminant in all our plague cultures. The contaminant, identified as *Ps. maltophilia*, consequently synthesized the APS that was found in *Y. pestis* cultivated in synthetic media. This is the first definitive demonstration of APS biosynthesis in bacteria. Purified cultures of *Y. pestis* produced no APS.

We are currently in the process of characterizing the APS contents from meat, agar, and *Pseudomonas*; after this is accomplished we will be able to complete our study on the chemical composition of APS of *Y. pestis*, after incorporation. We feel that it is a strange coincidence that APS are specifically incorporated into *Y. pestis* cells and that there is a specific and constant contamination problem that exists in our laboratory with an organism that has the unique ability to synthesize APS. Whether than the fact that the APS are specifically bound to *Y. pestis* cells and requires trichloroacetic acid extraction procedure to remove it, the details of its role remain unclear. Fig. 2 shows the colloidal thorium precipitate approach to visualizing the APS on the capsules surface of cells by electron microscopy. From this data one projects that the APS has a structural role perhaps as a microcapsule that transverses the capsule to cell wall. The nature of the chemical contents of the entities comprising the APS fraction supports this intention (Table VIII and Figs. 2 and 3). These data along with the data now unfolding in this laboratory, supports the hypothetic model for envelope structure of *Y. pestis* shown in Fig. 4.

Not only is this data showing structural evidence for APS in cell envelope and specific requirement properties by *Y. pestis* cells, it logically suggests that APS has a functional role as well. Consequently, lethality tests were conducted to determine if the APs influenced pathogenic or invasive properties in *Y. pestis* cells adapted to laboratory media. Experiments with *Y. pestis* were conducted with the common and selective contaminants, *Ps. maltophilia* 195, or commercially obtained *Ps. maltophilia* ATCC strain, or their APS products. These data are shown in Tables VIII and IX. The data show that *Y. pestis* with *Ps. maltophilia* 195, natural contaminant of *Y. pestis* cultures in this laboratory, or its autolysate, are effective in rendering the preparations more lethal in mice.

The curious point in this laboratory is the occurrence of *Ps. maltophilia* as a contaminant in our cultures.

The specific points are:

1. its the only contaminant detected in *Y. pestis* cultures.
2. we have detected it in cultures maintained in this laboratory as well as others but only after successive subcultivation.
3. its the only bacterium for which APS are found (although yet unpublished).

4. that *Y. pestis* seeks APS from culture media.
5. that Kitasato first reported motile bacteria in *Y. pestis* cultures (Bact. Rev. *40* #3 Sept. 1976, p. 633).

It is very difficult to initially show *Ps. maltophilia* in *Y. pestis* cultures, especially when *Ps. maltophilia* is held in very low percentages during cultivation. I feel confident that there is a relationship between *Pseudomonas* and *Yersinia* and I am committed to proving it. In our laboratory we feel that Kitasato's original description of a typical plague isolate was the most accurate.

The discovery of *Ps. maltophilia* in *Y. pestis* cultures compounded with the occurrence of APS, disrupted our research program for over one year during the tenure of United States Army Research Support. An additional year will be essentially lost before other sources of funding will be obtained. We have a wealth of data concerned with the plague diseases, much of which is presently disarticulated but it is only a matter of time before we acquire the necessary information and assemble the puzzle.

We feel that we have a unique system for studying at least one aspect of the complicated invasive and pathogenic properties of truly a unique bacterium, *Yersinia pestis*. These types of studies may appear to be afar from the original intent to chemo- or sero-type plague strain. But it is exactly these types of studies that will eventually reveal the route for which typing will be accomplished. We are convinced from these studies that typing of *Y. pestis* strains will not be accomplished by a simple exploration of somatic cellular entities.

Table I.

Molar compositions of Lipopolysaccharides (LPS) of four CDC strains of *Y. pestis*, nanomoles per mg.

LPS Fraction	Nanomoles per mg lipopolysaccharide[2]					
	KDO	Total Fatty Acids	β-hydroxy-myristate	Total Carbohydrate[2]	Total Amino Sugars	GlcN + GlcN 6P
28 C A1122 supernatant	53	29	12	608	139	130
28 C A1122 precipitate	78	148	107	801	299	299
37 C A1122 supernatant	98	12	4	1635	321	269
37 C A1122 precipitate	--	--	--	---	---	---
28 C Nairobi supernatant	45	57	20	2414	216	146
28 C Nairobi precipitate	22	41	37	895	115	105
37 C Nairobi supernatant	53	98	20	1503	415	272
37 C Nairobi precipitate	39	94	45	602	128	103
28 C Exu-5 supernatant	34	33	16	890	268	147
28 C Exu-5 precipitate	124	303	266	1669	148	130
37 C Exu-5 supernatant	78	74	61	884	89	77
37 C Exu-5 precipitate	--	--	--	---	---	---
28 C 195/P supernatant	122	T	T	---	526	524
28 C 195/P precipitate	--	--	--	---	---	---
37 C 195/P supernatant	296	T	0	718	185	165
37 C 195/P precipitate	284	381	344	1486	317	317

[1]Extracted by hot phenol-H_2O procedure

[2]Colorimetric determination based on glucose.

Abbreviations: GlcN, glucosamine; GlcN-6P, glucosamine-6-phosphate, T, trace.

Table II.

Lipopolysaccharide (LPS) yields and sugar composition from four CDC strains of *Y. pestis*.

LPS Fraction	Yield (% Dry Cell Weight)	Sugar Composition (Mole %)					
		AraN	Man	Gal	Glc	DD Hep	LD Hep
28 C A1122 supernatant	1.7	21.8	1.4	13.2	19.6	9.6	34.3
28 C A1122 precipitate	0.5	3.4	3.9	17.0	25.7	10.7	39.3
37 C A1122 supernatant	0.1	4.5	0.2	17.3	27.3	12.1	38.7
37 C A1122 precipitate	0.7	1.7	1.0	29.0	43.5	5.6	19.1
28 C Nairobi supernatant	3.7	8.9	0	T	83.1	2.4	5.6
28 C Nairobi precipitate	0.4	0.1	0.3	0	90.6	2.0	7.0
37 C Nairobi supernatant	0.1	T	42.4	23.2	25.9	2.4	6.1
37 C Nairobi precipitate	1.4	11.9	27.1	0.6	55.9	2.3	2.3
28 C Exu-5 supernatant	0.6	37.9	T	16.5	26.3	4.5	14.7
28 C Exu-5 precipitate	0.5	1.9	0.5	16.0	28.2	11.7	41.7
37 C Exu-5 supernatant	0.1	15.8	1.4	17.6	31.1	8.3	25.7
37 C Exu-5 precipitate	0.2	3.8	2.7	16.5	42.4	7.0	27.5
28 C 195/P supernatant	0.1	0	0	18.6	64.3	5.7	11.4
28 C 195/P precipitate	0.1	T	0	24.8	59.8	10.3	5.1
37 C 195/P supernatant	0.1	7.5	2.8	14.0	25.2	12.1	38.3
37 C 195/P precipitate	0.4	1.6	2.4	16.9	29.8	12.9	36.3

[1]Extracted by hot phenol-H_2O procedure.

Abbreviations: AraN, Arabinosamine; Man, Mannose; Gal, galactose; Glc, glucose; DD-Hep, DD-heptose; LD-Hep, LD-heptose.

Table III.

Amino sugar composition of Lipopolysaccharide (LPS) from four CDC strains of *Y. pestis*.[1]

LPS Fraction[2]	Nanomoles per mg lipopolysaccharide					
	GlcN 6P	GlcN	GalN	6-deoxy GlcN	6-deoxy GalN	EthN
28 C A1122 supernatant	4	126	1	0	8	4
28 C A1122 precipitate	84	215	0	0	0	0
37 C A1122 supernatant	2	267	35	4	13	0
37 C A1122 precipitate	--	---	--	--	--	-
28 C Nairobi supernatnat	6	140	37	27	6	0
28 C Nairobi precipitate	27	78	0	0	10	0
37 C Nairobi supernatant	32	240	133	0	10	0
37 C Nairobi precipitate	33	70	8	0	17	0
28 C Exu-5 supernatant	5	142	69	52	0	1
28 C Exu-5 precipitate	0	130	12	0	6	0
37 C Exu-5 supernatant	12	65	1	0	11	0
37 C Exu-5 precipitate	--	---	--	--	--	-
28 C 195/P supernatant	152	372	0	0	2	3
28 C 195/P precipitate	--	---	--	--	--	-
37 C 195/P supernatant	0	165	23	0	7	0
37 C 195/P precipitate	67	250	0	0	0	6

[1]Automated analysis by M. Yaguchi.

[2]Extracted by hot phenol-H_2O procedure.

Abbreviations: GlcN-6P, glucosamine-6-phosphate; GlcN, glucosamine; GalN, galactosamine; 6-deoxy GlcN, 6-deoxy glucosamine; 6-deoxy GalN, 6-deoxy galactosamine; EthN, Ethanolamine.

Table IV.

Fatty acid composition of lipopolysaccharides of four CDC strains of *Y. pestis*, nanomoles per mg.[1]

	Ester linked						Amide linked					
	Hexadecanoic acid	Hexadecenoic acid	Octadecanoic acid	Octadecenoic acid	β-Hydroxytetradecanoic acid	Others[2]	Hexadecanoic acid	Hexadecenoic acid	Octadecanoic acid	Octadecenoic acid	β-Hydroxytetradecanoic acid	Others[2]
28 C A 1122 supernatant	3	1	2	2	3	0	2	1	1	2	12	0
28 C A 1122 precipitate	2	19	1	1	37	8	2	1	1	1	70	7
37 C A 1122 supernatant	2	0	2	3	1	0	1	T	1	1	1	0
37 C A 1122 precipitate[3]	-	--	-	-	P	-	-	-	-	-	P	-
28 C Nairobi supernatant	2	2	0	0	7	29	1	0	0	0	14	0
28 C Nairobi precipitate	T	2	0	0	15	0	0	0	0	0	23	0
37 C Nairobi supernatant	7	1	1	1	13	37	T	1	0	0	32	2
37 C Nairobi precipitate	7	2	2	3	12	21	3	1	1	1	35	4
28 C Exu-5 supernatant	1	1	1	1	3	0	2	1	0	0	7	15
28 C Exu-5 precipitate	2	11	1	1	97	12	3	0	0	0	168	7
37 C Exu-5 supernatant	3	2	1	3	14	2	1	0	0	0	47	1
37 C Exu-5 precipitate[3]	-	--	-	-	P	--	-	-	-	-	P	--
28 C 195/P supernatnat[3]	-	--	-	-	P	--	-	-	-	-	P	--
28 C 195/P precipitate[3]	-	--	-	-	P	--	-	-	-	-	P	--
37 C 195/P supernatant	1	0	0	0	0	T	0	0	0	0	0	0
37 C 195/P precipitate	6	4	1	1	116	13	4	0	0	0	226	8

[1]Determined by GLC.
[2]As hexadecanoic acid.
[3]Quantities of sample were insufficient for quantitation. "P" signifies "present".

Table V.

Molar % composition of rough-lipopolysaccharides (LPS) of four strains of *Y. pestis*.

LPS Fraction[1]	Rhamnose and fucose	Ribose	AraN[2]	Man	Gal	Glc	DD-Hep	DL-Hep	GlcN
A1122	0	0	0	0	0	31	10	4.8	P
Nairobi	16.1	P	0	9.1	34.3	4.6	7.9	27.9	P
Exu-5	0	0	0	0	25.3	31	0	43.7	P
195/P	0	1.6	0	0	9.0	30	3.8	30	25.9

[1] Extraction by petroleum ether-phenol-chloroform method.

[2] Abbreviations: AraN, Arabinosamine; Man, Mannose; Gal, galactose; Glc, glucose; DD-Hep, DD-heptose; LD-Hep, LD-heptose; GlcN, glucosamine.

Table VI.

Composition of lipopolysaccharide (LPS) of *Y. pestis* pestoides number 36.

LPS Fraction	Total Carbohydrate	Total Hexosamine	Total KDO	Total Deoxysugar	Man[3]	Gal	Glc	DL-Hep	Glc-N
R-LPS[1]	----	----	---	----	---	12.8	34.5	51.7	0
SS-LPS[2]	18.4	0.5	1.5	18.4	5.1	11.9	36.0	17.7	0
SS-LPS[2]	17.4	1.8	4.8	2.3	0.9	8.4	22.2	18.4	45.6

[1]Rough-LPS extracted by petroleum ether-phenol-chloroform method.

[2]Smooth supernatant (SS-LPS)-and smooth-pellet (SP-LPS) extracted by hot phenol-H_2O method and separated by centrifugation.

[3]Abbreviations: Man, Mannose; Gal, galactose; Glc, glucose; LD-Hep, LD-heptose; Glc-N, glucosamine.

Table VII. Composition of Hydroxylapatite Fractions of acidic polysaccharides of *Y. pestis* 195/P.

	Percent Carbohydrate[a]	gal	glc	man[b]	Percent Uronic Acid[c]	Percent Protein[d]	Percent Phosphate	Percent Sulfate
HA I	74	100	-	-	1.8	17*	1.7	0.5
HA II	72	70	25	5	2.8	11	1.3	1.2
HA III	68	45	43	12	2.8	--	2.8	2.2
HA IV	54	20	65	15	7.0	13	nil	3.4
HA V	43	---	67	33	7.3	10	0.5	7.6
HA VI	37	---	67	33	9.7	9	0.9	8.0

[a] phenol-sulfuric acid assay; measured as galactose.

[b] measured as acetate derivatives by GLC

[c] measured as glucuronic acid.

[d] calculated from amino acid analyses data.

*HA I contained five to seven percent Fraction 1 protein in addition to the other protein.

Table VIII.

Lethality tests with laboratory adapted[1] *Y. pestis* and *Ps. maltophilia* cells or acidic polysaccharides from *Ps. maltophilia*.

Hours Post Injection	*Y. pestis* 195/p 3.54 x 10^7	*Ps. maltophilia* 195/P 1.3 x 10^8	Synthetic mixture mixed prior to inoculation 3.28 x 10^7 *Y. pestis* + 2.3 x 10^7 *Ps. maltophilia* 175/P	Natural mixture of *Ps. maltophilia* in stock *Y. pestis* 195/P; 4.3 x 10^7 organism
15	0/5*	0/5	3/5	5/5
18	0/5	0/5	4/5	
24	1/5	0/5	5/5	
31	2/5	0/5		
41	3/5	0/5		
50	4/5	0/5		
90	5/5	0/5		

[1]Laboratory adapted strains refers to the organism maintained on tryp-ticase soy agar for an extended period of time to empter the lethality of the test organism.

*Numbers represent total dead/number injected.

Table IX. Lethality test in mice with *Y. pestis* 195/P cultivated in trypticase soy broth enriched with autolysates of *Ps. maltophilia* ATCC or 195*.

	Sterile Controls		Trial 1			Trial 2			Trial 3			Total		
Hours Post Challenge	T-Soy + *Pseudomonas*-195 autolysate	T-Soy + *Pseudomonas*-ATCC autolysate	T-Soy	T-Soy + ATCC autolysate	T-Soy + 195 autolysate	T-Soy	T-Soy + ATCC autolysate	T-Soy + 195 autolysate	T-Soy	T-Soy + ATCC autolysate	T-Soy + 195 autolysate	T-Soy	T-Soy + ATCC autolysate	T-Soy + 195 autolysate
24	0/5	0/5	0/7	0/7	0/7	0/10	0/10	0/10	0/12	0/12	0/12	0/29	0/29	0/29
48	0/5	0/5	4/7	0/7	7/7	0/10	1/10	3/10	0/12	1/12	5/12	4/29	2/29	15/29
72	0/5	0/5	5/7	4/7		1/10	3/10	9/10	3/12	2/12	12/12	9/29	9/29	28/29
96	0/5	0/5	6/7	5/7		4/10	5/10	10/10	8/12	6/12		18/29	16/29	29/29
120	0/5	0/5	7/7	6/7		5/10	6/10		9/12	8/12		21/19	21/29	
144	0/5	0/5		6/7		7/10	8/10		12/12	10/12		26/29	24/29	
168	0/5	0/5		7/7		8/10	8/10			10/12		27/29	25/29	
Number Organisms Injected	0	0	6.1×10^6	5.8×10^6	4.1×10^6	1.5×10^6	2.1×10^6	1.5×10^6	2.6×10^5	3.1×10^5	2.10×10^5			
Mean Death Time			68.6	96	48	117	99	67.2	104	109	62	98.7	98.9	60.4

Ps. maltophilia 195 is that isolated as natural contaminant first isolated from *Y. pestis* 195/P culture.

Number in ratios represent total dead/number injected.

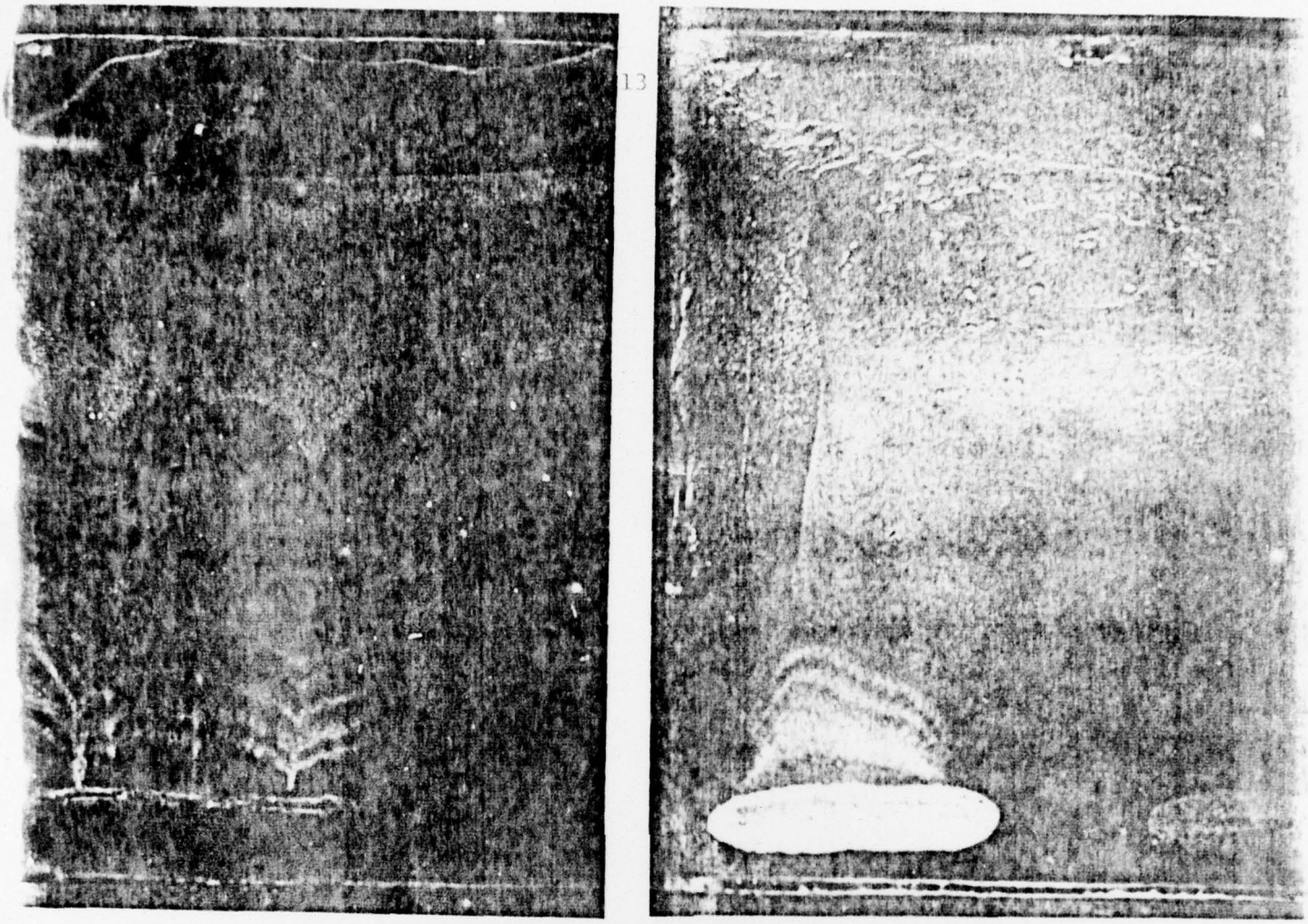
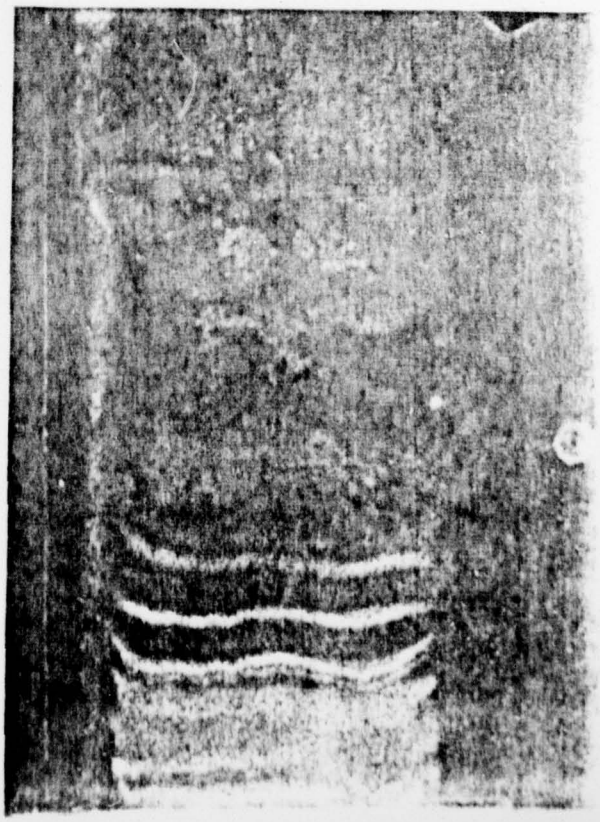


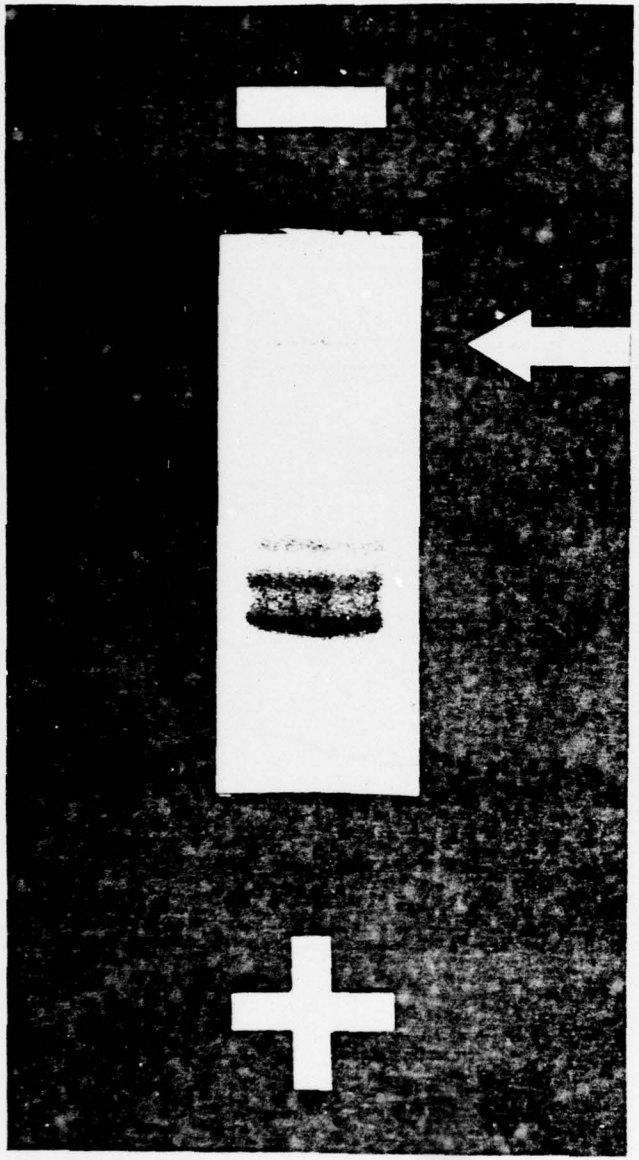
Fig. 1

Thin-layer chromatography of lipopolysaccharide preparations from pestoides, A1122, and 195/P reading clockwise. TLC plates were coated with Biosil A and developed in $ChCl_3$; MeOH; H_2O; NH_4OH (100:50:8:4). Compounds were usualized with I_2O vapors.

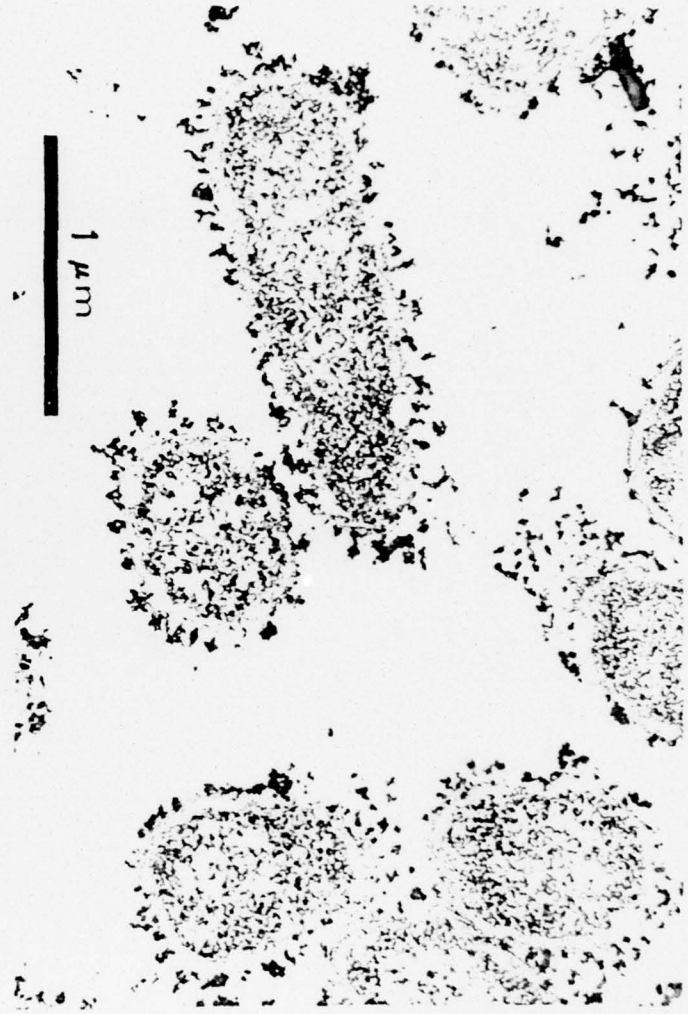


A

Fig. 2



Radioautogram of Hydroxylapaptite fraction HAV electrophoresed on cellulose acetate in 0.1N HCl. Fraction labeled with ^{35}S-Methionine.



Electron micrograph of colloidal thorium precipitated on acidic polysaccharide extending from cell surface

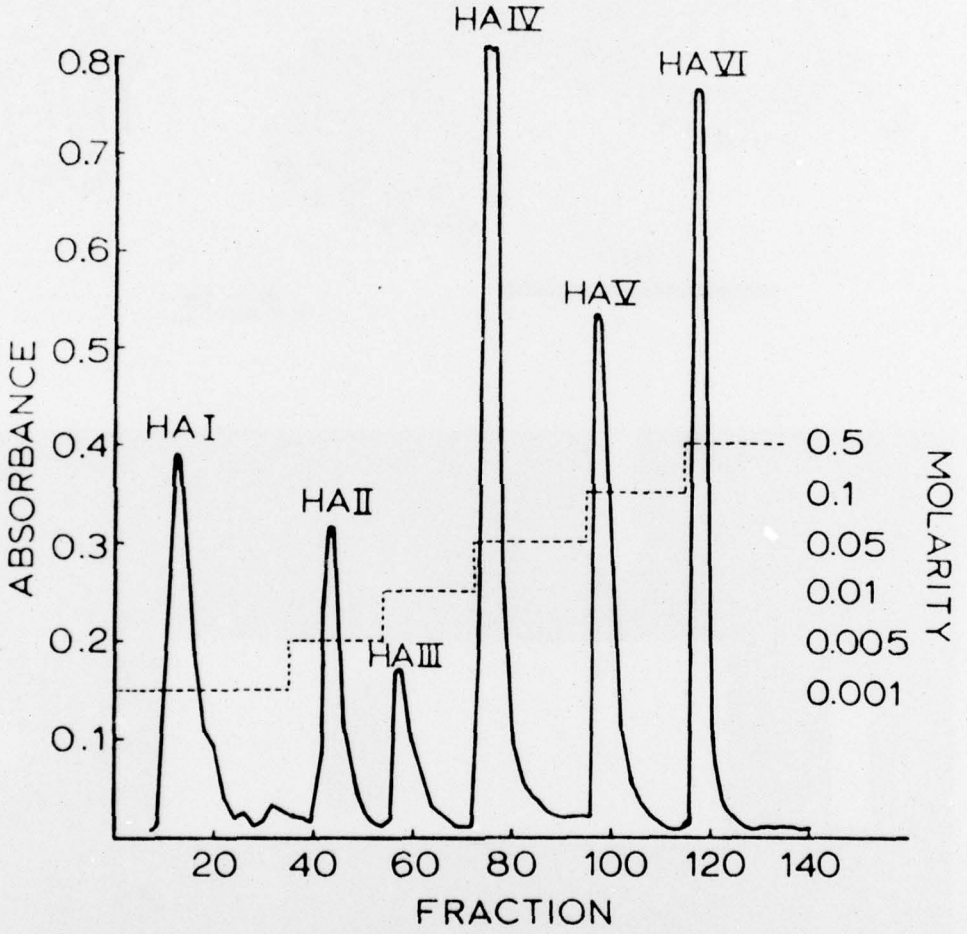
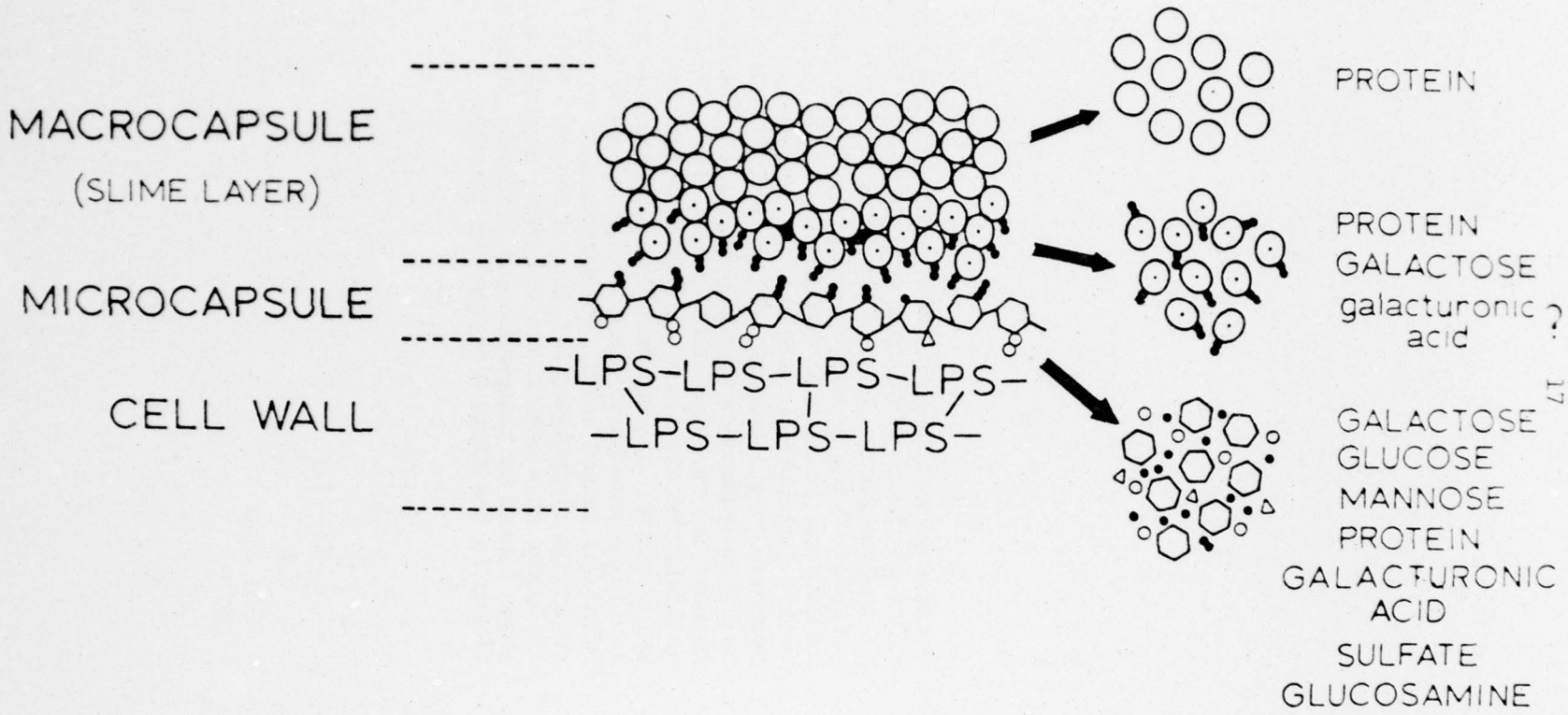


Fig. 3

Family of acidic polysaccharides obtained from sephadex column and fractionated on a hydroxylapatite column.

Fig. 4. A model postulating the structural relationships of macromolecules of the envelope-cell wall complex. The simple proteins of isoelectric point 4.85 are located in the most exterior portions of the envelope complex, with the glycoproteins of isoelectric point 4.65 located in closer proximity to the cell wall. Carbohydrate moieties are represented and shown to be associated with the chemically similar high molecular weight acidic polysaccharides occupying an intermediate location between the envelope and the cell wall. Interior to the high molecular weight acidic polysaccharides are low molecular weight acidic polysaccharides which are structurally similar to lipopolysaccharide O-antigens and serve to connect the high molecular weight acidic polysaccharides to the outer core region of lipopolysaccharides in the cell wall. Adjacent to the outer core region are the inner core LPS building blocks shown linked by phosphodiester bridges. These are in turn linked to the lipid A portion of the LPS.

MODEL
STRUCTURE
ENVELOPE
YERSINIA PESTIS



B. Bibliography of Publications.

1. Tornabene, Thomas G. Lipid composition of selected strains of *Yersinia pestis* and *Yersinia pseudotuberculosis*. Biochim. Biophys. Acta. *306* (1973) 173-185.

2. Bennett, Larry G., and Thomas G. Tornabene. Characterization of the antigenic subunits of the envelope protein of *Yersinia pestis*. J. Bacteriol. *117* (1974) 48-55.

3. Vulliet, Phillip, Sandford P. Markey, and Thomas G. Tornabene. Identification of methoxyester artifacts produced by methanolic-HCl solvolysis of the cyclorpopane fatty acids of genus *Yersinia*. Biochim. Biophys. Acta. *348* (1974) 299-301.

4. Hartley, J. L., Gordon A. Adams, and Thomas G. Tornabene. Chemical and physical properties of lipopolysaccharide of *Yersinia pestis* J. Bacteriol. *118* (1974) 848-854.

Papers in preparation:

1. Lipids, glycolipids and lipoproteins of *Pseudomonas maltophilia*.
2. Acidic polysaccharides of *Pseudomonas maltophilia*.
3. Acidic polysaccharides of *Y. pestis*.
4. Invasive and pathogenic properties of *Y. pestis*.

C. Personnel.

1. Larry G. Bennett, graduate student; received Ph.D., January 1975.
2. James L. Hartley, graduate student; received Ph.D., June 1975.
3. Carol Manweiler, graduate student; received M.S., August 1976.
4. Dr. Gordon Adams, Retired National Research Council of Canada, Ottawa, Visiting Professor.
5. Dr. Robbyn Happel, Post-doctoral fellow.